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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/11, 15/62, 15/85, A61K 67/27, 48/00

A1

(11) International Publication Number: WO 97/11168

(43) International Publication Date: 27 March 1997 (27.03.97)

(21) International Application Number: PCT/AU96/00595

(22) International Filing Date: 20 September 1996 (20.09.96)

(30) Priority Data:
PN 5596 22 September 1995 (22.09.95) AU
PO 1295 26 July 1996 (26.07.96) AU

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: PROMOTER AND USES THEREOF

(57) Abstract

The present invention relates to an isolated nucleic acid fragment including an intercellular adhesion molecule 2 ("ICAM-2") promoter or functionally active part thereof for expression of transgenes in endothelial cells. The present invention also relates to recombinant nucleic acid molecules including the promoter and transgenic animals capable of endothelial expression of introduced genes.

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PROMOTER AND USES THEREOF

The present invention relates to the expression of transgenes in the endothelium.

Consistent endothelial cell-specific expression of transgenes in adult animals has proved difficult to achieve. For example, attempts to target gene expression to the endothelium of transgenic mice have met with limited success. A 1.2 kb 5'-flanking region of the murine *Tie2* gene was sufficient to confer endothelium-specific expression of the reporter gene *lacZ* in transgenic mouse embryos but not in adult mice (Schlaeger *et al.*, Development 121 : 1089-1098, 1995). The human von Willebrand factor gene 5'-flanking region and first exon, shown to support high-level and specific endothelial cell expression *in vitro*, expressed *lacZ* in only a subpopulation of endothelial cells *in vivo* and not in the vascular beds of various organs examined (Aird *et al.*, Proc. Natl. Acad. Sci. USA 92 : 4567-4571, 1995). More promising results were obtained using the murine preproendothelin promoter to express either luciferase or a lipid-peroxidating enzyme in transgenic mice (Harats *et al.*, J. Clin. Invest. 95 : 1335-1344, 1995). However, expression of the transgenes was not confined to vascular endothelium, but was also present in arterial smooth muscle, and selected epithelium. Moreover, the level of expression ranged from high in arteries to low in veins and capillaries, and there was significant variation in expression both between and within organs. Thus, it is not truly endothelium-specific, and its pattern of expression in the vasculature is highly variable.

McCurry *et al.* Nature Medicine *1* : 423-427 (1995) describe a system involving the passive transfer of CD59 from erythrocytes to vascular endothelial cells. They demonstrate that the presence of CD59 prolongs the survival of porcine hearts transplanted into baboons. However, their model is limited by the transient nature of CD59 expression.

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In a first aspect, the present invention provides an isolated nucleic acid fragment including an intercellular adhesion molecule 2 ("ICAM-2") promoter or functionally active part thereof for expression of transgenes in endothelial cells. Preferably the ICAM-2 promoter or functionally active part thereof is 250-400

base pairs (bp) in length and corresponds to at least part of the 5' flanking region of the human ICAM-2 gene. More preferably the ICAM-2 promoter or functionally active part thereof is an approximately 336 bp *StyI* nucleic acid fragment. Most preferably the ICAM-2 promoter or functionally active part thereof includes the nucleic acid sequence

5'CCAGGCATGACTCCAACAATGCATCCCATGGGATTTGGGGTTCCCCAGATCT
GGGGCTTGTAGGCCTGACTCTCCCCTGTGCACACGTCTCATACACGCATG
CGTGCACCCATTGCCTGCCCCGCCCCTTGCACAGGGAGTCAGCAGGGAGG
ACTGGGTTATGCCCTGCTTATCAGCAGCTTCCCAGCTTCCTCTGCCTGGA
TTCTTAGAGGCCTGGGGTCCTAGAACGAGCTGGTGCACGTGGCTTCCCAA
AGATCTCTCAGATAATGAGAGGAAATGCAGTCATCAGTTTGCAGAAGGCT
AGGGATTCTGGGCCATAGCTCAGACCTGCGCCCACCATCTCCCTCCAGGC
AGCCCTTGGCTGGTCCCTGCGAGCCCGTGGAGACTGCCAGAGATGTC 3'

functionally active parts thereof and sequences substantially homologous thereto.

In a second aspect, the present invention provides a recombinant nucleic acid molecule, capable of being expressed in endothelial cells, said molecule including the following operatively linked components:

a promoter as hereinbefore described,

an intron,

one or more genes or functionally active parts thereof, and

a termination signal.

The intron may be of any suitable type. Preferably the intron is a hybrid or 'universal' intron, such as that described by Choi et al., Molec. Cell. Biol. 11:3070-3074, 1991, the entire disclosure of which is incorporated herein by reference.

The one or more genes may be any genes for which endothelial expression is desired. Preferably the one or more genes encode complement regulatory factors. The one or more genes may be selected from the group consisting of CD59, CD46 and CD55, and modified versions thereof. For example the one or more genes may be selected from the group consisting of human CD59, CD46 and CD55 cDNA. The gene may be a hybrid of two or more genes. The gene may be a hybrid of CD55 and CD59, and modified versions thereof, for example CD55-internal ribosome entry site ("IRES")-CD59. The gene may be a hybrid of

CD46, CD55 and CD59, and modified versions thereof. The gene may be modified to facilitate expression. For example the transmembrane and cytoplasmic domains of CD46 may be replaced by a glycosylphosphatidylinositol (GPI) tail.

The termination signal may be of any suitable type. Preferably the termination signal is a polyadenylation signal, more preferably the SV40 early polyadenylation signal.

In a further aspect, the present invention provides a vector including the recombinant nucleic acid molecule as hereinbefore described.

In a still further aspect, the present invention provides a method of expressing one or more genes in the endothelium of an animal, which method includes introducing into said animal one or more constructs, each construct including a promoter as hereinbefore described operatively linked to said one or more genes. Preferably the construct includes a recombinant nucleic acid molecule as hereinbefore described. Preferably introduction of said construct into said animal is by means of liposomal delivery systems or viral vectors, for example as described in Evans, R. et al., Annals N.Y. Acad. Sci. 716:257-264, 1994, the entire disclosure of which is incorporated herein by reference. Alternatively said construct may be introduced into a precursor of said animal, for example a fertilized egg, by means of standard microinjection techniques which are well known to those skilled in the art.

In a still further aspect the present invention provides a transgenic animal capable of endothelial expression of one or more genes, said animal having introduced therein one or more constructs, each construct including a promoter as hereinbefore described operatively linked to said one or more genes. Preferably the construct includes a recombinant nucleic acid molecule as hereinbefore described. Preferably introduction of said construct into said animal is by means of liposomal delivery systems or viral vectors, for example as described in Evans, R. et al., 1994. Alternatively said construct may be introduced into a precursor of said animal, for example a fertilized egg, by means of standard microinjection techniques which are well known to those skilled in the art.

In a still further aspect the present invention provides a method of prolonging the survival of organs grafted from an animal, said method including

introducing into said animal one or more constructs, each construct including a promoter as hereinbefore described operatively linked to one or more genes, the expression of which prolongs transplant survival. Preferably the construct includes a recombinant nucleic acid molecule as hereinbefore described. Preferably introduction of said construct into said animal is by means of liposomal delivery systems or viral vectors, for example as described in Evans, R. et al., 1994. Alternatively said construct may be introduced into a precursor of said animal, for example a fertilized egg, by means of standard microinjection techniques which are well known to those skilled in the art.

The organ to be transplanted may be of any suitable type. In a preferred aspect of the present invention, the grafted organ may be a heart.

The present invention will now be more fully described with reference to the accompanying Example and Figures. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the Figures

Fig. 1 *a*, Sequence of the 5'-flanking region of the human ICAM-2 gene amplified by PCR. Numbering is relative to the A of the ICAM-2 start codon (underlined). Putative regulatory elements are in bold type and underlined, and an inverted GCAAT motif is boxed. The 5' end of an ICAM-2 cDNA is indicated by the symbol # above the sequence. *b*, Structure of the 2.5-kb *Bss*HII microinjection construct, showing the ICAM-2 promoter, universal intron (UI), CD59 cDNA, and SV40 early polyadenylation signal sequence (poly(A)). The location of a ^{32}P-labelled PCR-generated probe used for dot blot analysis is shown. *c*, Determination of transgene copy number by dot blotting. The hybridization signal generated by known quantities of the microinjection construct (two row - indicated as number of copies per genome equivalent) was compared with the signals from genomic DNA of founders 237-4 and 237-7, their offspring (A19 and A21, respectively), and wild type (WT). Hybridization with a GAPDH probe was used to correct for differences in the amount of DNA blotted.

Fig. 2 Expression of human CD59 on granulocytes, as determined by flow cytometry. Transgenic founder 237-7 is compared with a non-transgenic littermate and with humans.

Fig. 3 Immunocytochemical detection of CD59 expression in the organs of transgenic mice and non-transgenic littermates. *a*, Kidney section (x 66) of transgenic mouse, showing strong staining of the endothelium of a glomerulus (g), intertubular sirusoids (is), and transverse section of a large vessel (v). *b*, Kidney (x 80) of control mouse. *c*, Heart (x 100) of transgenic mouse. Staining can be seen on a large vessel and on all capillaries. *d*, Heart (x 100) of control mouse. *e*, Lung (x 66) of transgenic mouse, showing specific staining of large vessel endothelium (v) and absence of staining on bronchioles (b). Strong staining is evident in the small vessel/alveoli region, although these tissues cannot be distinguished clearly due to compression. *f*, Lung (x 66) of control mouse. *g*, Liver (x 66) of transgenic mouse, showing specific staining of all vascular endothelium including the central vein (cv). *h*, Liver (x 66) of control mouse. *i*, Pancreas (x 66) of transgenic mouse, showing specific staining of vessels and absence of staining on islets (i). *j*, Pancreas (x 50) of control mouse.

EXAMPLE 1: CD59

Methods

Transgene construction

The 5'-flanking region (positions -394 to +5 relative to the A of the start codon, Fig. 1a) of the human ICAM-2 gene was amplified from genomic DNA by polymerase chain reaction (PCR) using primers (5' CCAGGCATGACTCCAACAATGC 3' and 5' CTGTAGAGACCGTCAGAGGTGC 3'). The 50-μl reaction contained 2 μg of human genomic DNA, 25 pmol of each primer, dNTPs at 200 μM, and 0.7 U of Taq DNA polymerase. The conditions for amplification were an initial 4 min. denaturation at 94°C followed by 35 cycles at 94°C for 60 sec, 60°C for 60 sec, and 72°C for 60 sec.

A 336-basepair fragment (-369 to -34) released from the PCR product by digestion with *Sty*I was blunt-ended using Klenow DNA polymerase and subcloned into the *Sma*I site of pBluescript II SK$^+$ (Stratagene Inc., La Jolla, CA) such that the upstream end of the ICAM-2 5'-flanking region was closest to the *Kpn*I site of the vector. This region was isolated as a *Kpn*I/blunt fragment from the resulting plasmid after sequential treatment with *Bam*HI, Klenow DNA polymerase, and *Kpn*I, and cloned into a 4.9-kb CD59 microinjection vector (described below) which had been treated with *Cla*I, Klenow DNA polymerase,

and *Kpn*I.

The CD59 microinjection vector contained a hybrid, or "universal" intron shown to increase gene expression in transgenic mice (Choi et al., 1991), a human CD59 cDNA, and the early polyadenylation signal from SV40, all cloned into the multiple cloning site of pBluescript II SK$^+$. The intron was cloned as a 243-basepair fragment obtained by *Cla*I/*Eco*RI digestion of a 254-basepair PCR product amplified from plasmid pXMT2 using the primers 5' CTCATCGATTGGGGTGAGTAC 3' (*Cla*I site underlined) and 5' AGTGAATTCCTGTGGAAGAGAAAGGC 3' (*Eco*RI site underlined). The CD59 cDNA was isolated from plasmid pMPZenSVNeo/CD59 (Somerville et al., Transplantation, 58:1430-1435, 1994, the entire disclosure of which is incorporated herein by reference) as a 1.2-kb *Xho*I fragment, to which *Eco*RI linkers were added before cloning. The SV40 early poly(A) fragment consisted of bases 3002-2452 of SV40 (Reddy et al., Nucleic Acids Res 1978; 5: 4195, the entire disclosure of which is incorporated herein by reference).

Generation of transgenic mice

The 2.5-kb microinjection construct was excised from the vector by digestion with *Bss*HII and separated by agarose gel electrophoresis, followed by purification through sequential Wizard (Promega, Madison, WI) and CHROMA SPIN 400 (CLONTECH, Palo Alto, CA) minicolumns. The purified fragment was microinjected at a concentration of 10 μg/ml in phosphate-buffered saline into fertilized CBA x C57/BL6 F2 mouse eggs. Progeny were screened after weaning by formamide low temperature (FoLT) PCR (Panaccio *et al.*, BioTechniques 14:238-243, 1993, the entire disclosure of which is incorporated herein by reference) of whole blood, collected by eye bleeding. Five microliters of blood were "solubilized" in 50% deionised formamide (75μl) at 95°C for 5 min. Fifteen microliters of the treated blood were used in a 50μl formamide low temperature PCR containing the following components: 10mM Tris-HC1 (pH 9.0), 50 mM KC1, 1.5 mM $MgC1_2$, 0.01% (w/v) gelatin, 0.1% (v/v) Triton X-100, 200 μM dNTPs, 20 pmol each of two primers, (5' CAAGTGTTGGAAGTTTGAGC 3' and 5' GCCCCTCAAACCCTCTTCGA 3') specific for the CD59 cDNA and 1-2U of Tth polymerase (Promega). The resulting 234-basepair product was resolved by electrophoresis in 2% agarose.

Transgene copy number and integrity were examined by dot blot and Southern analysis of DNA extracted from tail tips of transgenic mice. Blots were hybridized with a ^{32}P-labelled probe generated by PCR of CD59 cDNA using the above primers. Hybridization with a probe for the glyceraldehyde phosphate dehydrogenase gene was used to correct for differences in the amount of DNA blotted.

Flow cytometric analysis

Peripheral blood leukocytes (PBLs) were isolated from mouse blood using standard techniques, and incubated with fluoresceinated mouse anti-human CD59 monoclonal antibody MEM-43 (Serotech, Oxford, England). Labelled cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Sunnyvale, CA), using human PBLs as a positive control. Granulocytes were gated following sequential staining with the rat anti-mouse granulocyte monoclonal antibody RB6-8C5 (Holmes, K.L. et al., J. Immun. 137:679-688, 1986, the entire disclosure of which is incorporated herein by reference), a biotinylated goat anti-rat Ig monoclonal antibody (Caltag, San Francisco, CA), and streptavidin-tricolor reagent (Caltag).

Immunohistochemistry

Fresh-frozen tissue sections (4 µm) were incubated successively with Tris-buffered saline (pH 7.4) containing 10% heat-inactivated sheep serum, FITC-conjugated MEM-43, and POD-conjugated anti-fluorescein Fab fragments (Boehringer Mannheim, Germany). Peroxidase staining was developed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma St Louis, MO). Sections were counterstained with haematoxylin. For staining of endothelial cells, the hamster anti-mouse PECAM-1 monoclonal antibody 2H8 (Bogen, S.A. et al., Amer, J. Pathol. 141:843-854, 1992, the entire disclosure of which is incorporated herein by reference) was used.

Results

Analysis of Transgenic mice

The 5'-flanking segment of the human ICAM-2 gene extending 369 basepairs upstream of the start codon was amplified by PCR. This region contains two motifs for GATA, three for ETS, and one each for Sp1 and CACCC (Fig. 1a).

A CD59 cDNA was cloned downstream of the ICAM-2 5'-flanking region (Fig. 1b).

Of 27 offspring generated by microinjection of the transgene into fertilised mouse oocytes, four were transgenic as determined by PCR analysis of peripheral blood. Flow cytometric analysis of white blood cells demonstrated that three of the four founders expressed CD59 (Fig. 2), at levels 7- to 10-fold higher than that of CD59 on human granulocytes. The majority of expressing cells, comprising 30-35% of total PBL, were identified as granulocytes (of which more than 90% are neutrophils) by double-staining with anti-CD59 and the granulocyte-specific monoclonal antibody RB6-8C5. High expression of CD59 was also observed on monocytes but not on lymphocytes.

Two of the expressors, 237-4 and 237-7, produced offspring when mated with wild-type mice. (The third expressor [223-4] did not mate and was euthanized for immunohistochemistry). From dot blot analyses of tail-tip DNA from founders and heterozygous F_1 offspring, transgene copy numbers for the 237-7 and 237-4 lines were estimated at 4 and 8, respectively (Fig. 1c). Southern blot analysis of genomic DNA restricted with *BglI*, which cuts the construct once, indicated head-to-tail integration of the transgene copies at distinct sites for each line.

Pattern of transgene expression in transplantable organs

The pattern and intensity of staining were similar in all three lines. Staining was confined to the vascular endothelium in all organs: in the kidney, on glomeruli, intertubular sinusoids, and large vessels (Fig. 3a); in the heart, on vessels of all sizes (Fig. 3c); in the liver, on all vessels, including the central vein (Fig. 3g); and in the pancreas, on vessels but not on islets. In the lung, there was specific staining of large vessel endothelium and absence of staining on bronchioles (Fig. 3e). Strong staining was also evident in the small vessel/alveoli region, although these tissues could not be distinguished clearly due to compression. No staining was seen in the tissues of a negative littermate (Fig. 3).

Identification of the stained regions as endothelium was confirmed by staining of serial sections with a monoclonal antibody to PECAM-1, which is a commonly-used marker for endothelial cells (Risau, W. FASEB J. 9, 926-935, 1995, the entire disclosure of which is incorporated herein by reference)

Further analysis of transgenic mice

The transgenic mice were further analysed using an *ex vivo* isolated perfused heart system. Hearts from transgenic and non-transgenic mice were removed, placed on a rig, and perfused with buffer. The hearts continued to beat and heart work was continuously measured. After a period of stabilisation, human plasma was added to the buffer reservoir and the effect on heart work was monitored.

The performance of control hearts rapidly deteriorated after addition of 6% human plasma, with work falling below 50% within 20 minutes. In contrast, the transgenic hearts were still functioning at over 80% of the initial work rate 50 minutes after plasma addition. Immunohistochemical analysis showed extensive damage and MAC deposition in control hearts, whereas transgenic hearts were morphologically intact and largely clean of MAC.

The high-level endothelial expression of CD59 driven by the ICAM-2 promoter protects mouse hearts from damage by human plasma and prolongs their function compared to non-transgenic controls. This is in contrast to the lack of prolongation of function seen when CD59 expression is driven by the mouse H-$2K^b$ promoter (H-$2K^b$ is an MHC Class I molecule expressed widely throughout the tissues; endothelial expression of CD59 in our lines of H-$2K^b$/CD59 transgenic mice was significantly lower than that in ICAM-2/CD59 transgenic mice).

Discussion

We report here the use of the human ICAM-2 promoter to target strong, constitutive gene expression to endothelial cells of transgenic mice, more particularly endothelial cells of heart, liver, lung, kidney and pancreas in transgenic mice. Specifically, we found that a 336-bp fragment from the 5' flanking region of the human ICAM-2 gene contained the necessary signals to target high-level transgene expression to endothelial tissue in transgenic mice. Unexpectedly, our results suggest that most or all of the signals necessary for tissue specificity reside within a very small (336-bp) promoter region. In a number of other studies it has been shown that much larger promoter fragments are often required to parallel the tissue-specific expression of endogenous genes. For example, another relatively short segment, the 733-bp von Willebrand factor promoter, did not function correctly *in vivo* despite its ability to support specific

endothelial cell expression *in vitro* (Aird *et al.*, 1995). In contrast, the ICAM-2 promoter targeted expression consistently to all blood vessels regardless of size or type in all organs examined (Fig. 3).

Although the expression of CD59 in the tissues of the transgenic mice mimicked that of ICAM-2 in humans, all three expressing lines exhibited high levels of CD59 on neutrophils (Fig. 2). This was unexpected given the lack of expression of ICAM-2 on resting human neutrophils (de Fougerolles *et al.*, J. Exp. Med. 174 : 253-267, 1991). Whilst applicant does not wish to be restricted by theory, it is possible that the cloned human promoter lacks a negative regulatory element. Alternatively, the ICAM-2 promoter may indeed be active in neutrophils, but the ICAM-2 synthesized may be stored internally and relocated to the cell surface upon activation, in a manner analogous to the storage and release of P-selectin and von Willebrand factor by endothelial cells.

The strong and specific staining for CD59 in all three expressing F_0 mice suggests that the ICAM-2 promoter functioned in a relatively position-independent manner. One cause of integration site effects is presumably promoter occlusion, i.e. transcriptional interference to the transgene promoter by polymerase molecules that have transcribed upstream genes. In this respect it is noteworthy that the upstream region of the human ICAM-2 promoter contains an inverted GCAAT motif (Fig. 1*a*). A promoter-proximal, inverted CCAAT box has been shown to signal transcription termination via a mechanism postulated to involve protein binding (Connelly and Manley, Molec. Cell. Biol. 9, 5254-5259, 1989), and the affinity of CCAAT-binding protein (CBP) is increased by mutation of the target site to GCAAT (Graves *et al.*, Cell 44:565-576, 1986). Whilst applicant does not wish to be restricted by theory, it is conceivable that the inverted GCAAT in the ICAM-2 promoter reduced promoter occlusion by blocking transcriptional readthrough.

The ICAM-2 promoter may be used to engineer vascular endothelium-specific expression of human complement inhibitors and other immunoregulatory molecules in transgenic pigs. The ICAM-2 promoter is unique among endothelium-specific promoters studied to date in that it provides a means of constitutively expressing CD59 and other molecules at high levels in all vascular endothelium. The specificity and strength of the ICAM-2 promoter makes it a

useful tool in fields including xenotransplantation, the study of vascular biology, and gene therapy in the treatment of cancer. These areas of medical research will benefit from an *in vivo* targeting system capable of producing high-level constitutive gene expression specifically in vascular endothelium. The study of human vascular diseases such as atherosclerosis will be advanced by the availability of transgenic mice overexpressing biologically important proteins throughout the vascular tree. The success of pig-to-human xenotransplantation is likely to hinge on the constitutive expression of human complement inhibitors and other immunoregulatory molecules on the vascular endothelium of the organs of transgenic pigs. Gene therapy to limit injury to non-malignant endothelium caused by anticancer agents is another area of keen interest. Retroviral and liposomal delivery systems may be complemented by the use of strong endothelium-specific promoters to express protective genes.

EXAMPLE 2: CD55 and CD59

Transgenic mice expressing both CD55 and CD59 were generated by coinjecting two constructs, with the ICAM-2 promoter driving either CD55 or CD59. Two constructs comprising the ICAM-2 promoter driving expression of either CD55 or CD59 cDNA were co-injected into fertilised mouse ova to generate transgenic mice. PCR analysis of genomic DNA of the offspring identified one mouse (FH304.2) in which both constructs were integrated. FACS analysis demonstrated that both CD55 and CD59 were expressed on neutrophils in FH304.2 at levels higher than that on human neutrophils. Breeding analysis of the FH304.2 line indicated that the transgenes were inherited as a single genetic unit. The results suggested that co-injection with ICAM-2 promoter-driven constructs was feasible as a relatively rapid method of generating transgenic animals expressing multiple transgenes. FH304.2 was bred to produce mice for an immunohistochemical survey of the tissue distribution of CD55 and CD59. Both transgenes were expressed in the typical ICAM-driven pattern i.e. very strong expression on vascular endothelium and neutrophils but not on most other cell types. We have tested hearts from these CD55/CD59 double transgenics on our *ex vivo* perfusion rig. The CD55/CD59 hearts were protected from injury by human plasma and functioned at more than 50% maximum work till the end of the experiment (45 minutes after plasma addition), in contrast to non-transgenic

hearts which stopped beating 5 to 10 minutes after plasma addition.

EXAMPLE 3: CD46

It was not possible to generate mice expressing human CD46 using a cDNA-based construct (0 expressors from 10 transgenics). We have used an alternative approach to express CD46 in mice by replacing the transmembrane and cytoplasmic domains of CD46 with a glycosyl-phosphatidylinositol (GPI) tail. 11 transgenic mice were generated and flow cytometric analysis showed that CD46 was expressed on the neutrophils of 3 of these mice, at levels up to 2.5 fold higher than that of CD46 on human neutrophils. Histological analysis of kidneys showed strong expression of CD46 on all vascular endothelium. Whilst applicant does not wish to be restricted by theory, it appears that inappropriate expression of the full-length molecule may have interfered with embryogenesis, perhaps via signalling involving the cytoplasmic tail.

EXAMPLE 4: CD55 and CD59

IRES stands for internal ribosome entry site, and is a means of getting translation of a downstream open reading frame in a polycistronic message. Eukaryotic transcripts are normally translated as follows: the ribosome binds to the 5' end of the mRNA and 'scans' along the message until it finds a start codon in good context; the following ORF is then translated and the ribosome dissociates irrespective of the presence of downstream ORFs. If however you have a single transcript containing two ORFs separated by an IRES, both can be translated. We generated transgenic mice expressing both CD55 and CD59 by coinjecting two constructs, with the ICAM-2 promoter driving either CD55 or CD59. For example, we used the ICAM-2 promoter to drive the expression of a CD55-IRES-CD59 message (i.e. distinct from both CD55 or CD59 alone). Transgenic mice expressing both proteins were generated, again with the expected pattern although CD59 was at a somewhat lower level than CD55, presumably due to lower efficiency of IRES-directed translation.

EXAMPLE 5: CD46, CD55 and CD59

We have generated transgenic mice by coinjecting three constructs, with the ICAM-2 promoter driving either CD46 (the modified version bearing a GPI membrane linkage rather than a transmembrane domain), CD55 or CD59. Six mice that express all three transgenes (on neutrophils) have been identified.

Finally, it is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

CLAIMS:

1. An isolated nucleic acid fragment including an intercellular adhesion molecule 2 ("ICAM-2") promoter or functionally active part thereof for expression of transgenes in endothelial cells.

2. An isolated nucleic acid fragment according to claim 1 wherein the ICAM-2 promoter or functionally active part thereof is 250-400 base pairs (bp) in length.

3. An isolated nucleic acid fragment according to claim 2 wherein the ICAM-2 promoter or functionally active part thereof corresponds to at least part of the 5' flanking region of the human ICAM-2 gene.

4. An isolated nucleic acid fragment according to claim 1 wherein the ICAM-2 promoter or functionally active part thereof is an approximately 336 bp *StyI* nucleic acid fragment.

5. An isolated nucleic acid fragment according to claim 1 wherein the ICAM-2 promoter or functionally active part thereof includes the nucleic acid sequence
5'CCAGGCATGACTCCAACAATGCATCCCATGGGATTTGGGGTTCCCCAGATCT
GGGGCTTGTAGGCCTGACTCTCCCCTGTGCACACGTCTCATACACGCATG
CGTGCACCCATTGCCTGCCCCGCCCCTTGCACAGGGAGTCAGCAGGGAGG
ACTGGGTTATGCCCTGCTTATCAGCAGCTTCCCAGCTTCCTCTGCCTGGA
TTCTTAGAGGCCTGGGGTCCTAGAACGAGCTGGTGCACGTGGCTTCCCAA
AGATCTCTCAGATAATGAGAGGAAATGCAGTCATCAGTTTGCAGAAGGCT
AGGGATTCTGGGCCATAGCTCAGACCTGCGCCCACCATCTCCCTCCAGGC
AGCCCTTGGCTGGTCCCTGCGAGCCCGTGGAGACTGCCAGAGATGTC 3'
functionally active parts thereof and sequences substantially homologous thereto.

6. A recombinant nucleic acid molecule, capable of being expressed in endothelial cells, said molecule including the following operatively linked components:

an ICAM-2 promoter or functionally active part thereof,
an intron,
one or more genes or functionally active parts thereof, and
a termination signal.

7. A recombinant nucleic acid molecule according to claim 6 wherein the one or more genes encode complement regulatory factors.

8. A recombinant nucleic acid molecule according to claim 7 wherein the one or more genes are selected from the group consisting of CD59, CD46 and CD55, and modified versions thereof.

9. A recombinant nucleic acid molecule according to claim 8 wherein the one or more genes is a hybrid of CD55 and CD59 or of CD46, CD55 and CD59, and modified versions thereof.

10. A recombinant nucleic acid vector according to claim 6 wherein the intron is a hybrid or universal intron and the termination signal is the SV40 early polyadenylation signal.

11. A vector including a recombinant nucleic acid molecule according to claim 6.

12. A method of expressing one or more genes in the endothelium of an animal, which method includes introducing into said animal one or more constructs, each construct including an ICAM-2 promoter or functionally active part thereof operatively linked to said one or more genes.

13. A method according to claim 12 wherein the construct includes a recombinant nucleic acid molecule according to claim 6.

14. A method according to claim 13 wherein the construct is introduced into said animal by means of liposomal delivery systems or viral vectors or into a precursor of said animal, for example a fertilized egg, by means of microinjection techniques.

15. A transgenic animal capable of endothelial expression of one or more genes, said animal having introduced therein one or more constructs, each construct including an ICAM-2 promoter or functionally active part thereof operatively linked to said one or more genes.

16. A transgenic animal according to claim 15 wherein the construct includes a recombinant nucleic acid molecule according to claim 6.

17. A transgenic animal according to claim 16 wherein the construct is introduced into said animal by means of liposomal delivery systems or viral vectors or into a precursor of said animal, for example a fertilized egg, by means of microinjection techniques.

18. A method of prolonging the survival of organs grafted from an animal, said method including introducing into said animal one or more constructs, each

construct including an ICAM-2 promoter or functionally active part thereof operatively linked to one or more genes, the expression of which prolongs transplant survival.

19. A method according to claim 18 wherein the construct includes a recombinant nucleic acid molecule according to claim 6.

20. A method according to claim 19 wherein the construct is introduced into said animal by means of liposomal delivery systems or viral vectors or into a precursor of said animal, for example a fertilized egg, by means of microinjection techniques.

21. A method according to claim 18 wherein the organ is a heart.

```
                                           StyI
-392 CCAGGCATGACTCCAACAATGCATCCCATGGGATTTGGGTTCCCAGATCT -343

-342 GGGGCTTGTAGGCCTGACTCTCCCCTGTGCACACGTCTCATACACGCATG -293

                            Sp1
-292 CGTGCACCCATTGCCTGCCCCGCCCCTTGCACAGGGAGTCAGCAGGGAGG -243

                       GATA       ETS       ETS
-242 ACTGGGTTATGCCCTGCTTATCAGCAGCTTCCCAGCTTCCTCTGCCTGGA -193

                                                     ETS
-192 TTCTTAGAGGCCTGGGGTCCTAGAACGAGCTGGTGCACGTGGCTTCCCAA -143

                 GATA
-142 AGATCTCTCAGATAATGAGAGGAAATGCAGTCATCAGTTTGCAGAAGGCT -93

                                            #
 -92 AGGGATTCTGGGCCATAGCTCAGACCTGCGCCCACCATCTCCCTCCAGGC -43

        StyI
 -42 AGCCCTTGGCTGGTCCCTGCGAGCCCGTGGAGACTGCCAGAGATGTC    +5
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FIG 1a

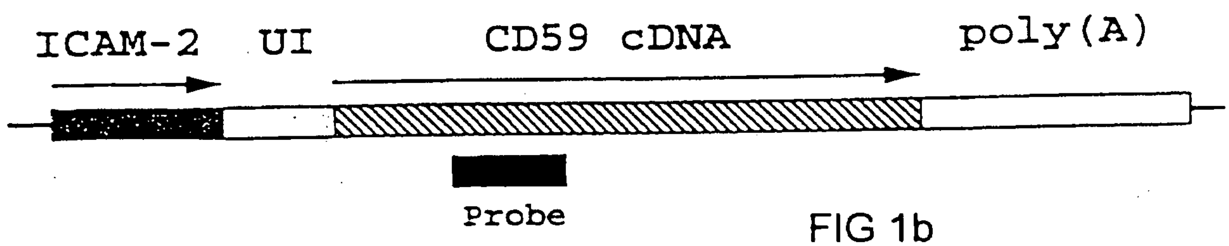

FIG 1b

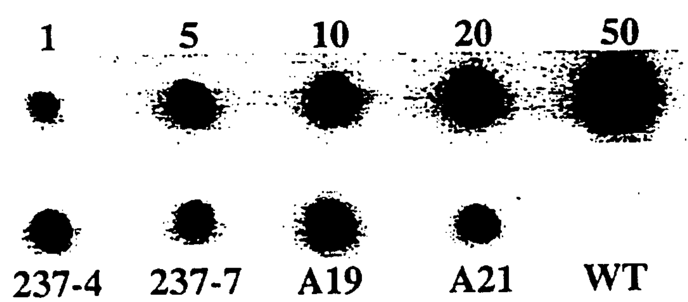

FIG 1c

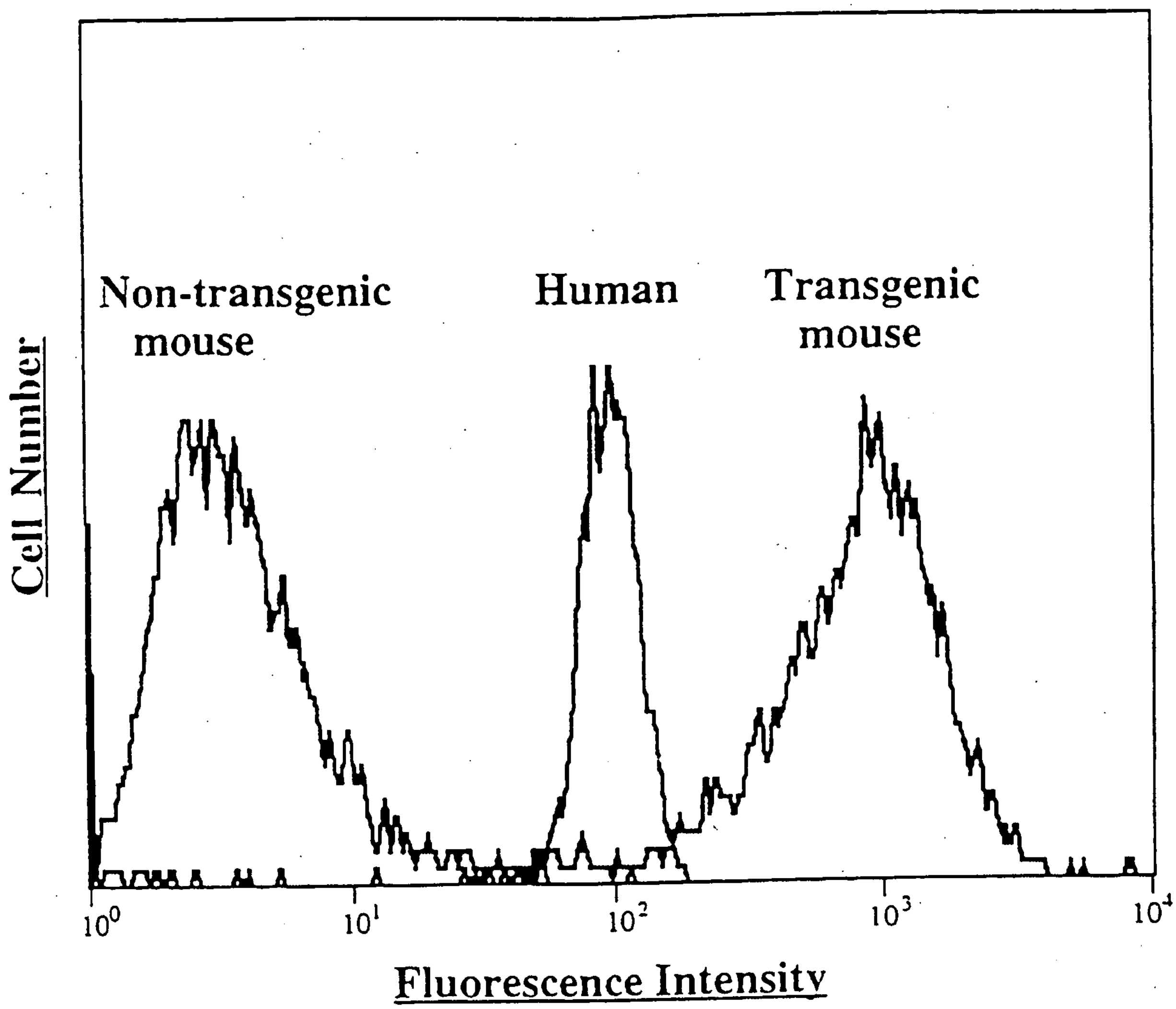
Non-transgenic mouse
Human
Transgenic mouse
Cell Number
10^0
10^1
10^2
10^3
10^4
Fluorescence Intensity

FIG 2

BEST AVAILABLE COPY

FIG 3a

FIG 3b

FIG 3c

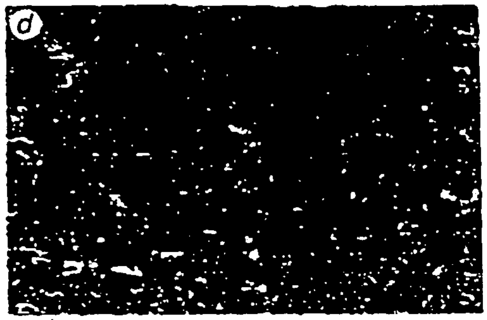

FIG 3d

FIG 3e

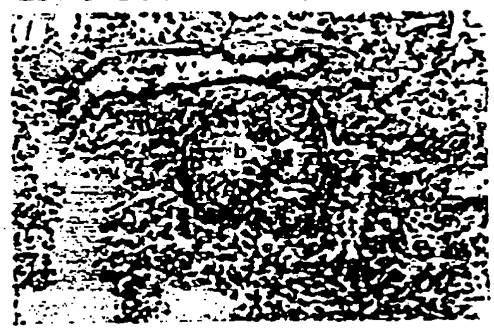

FIG 3f

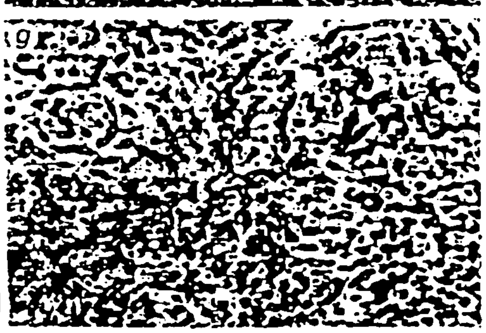

FIG 3g

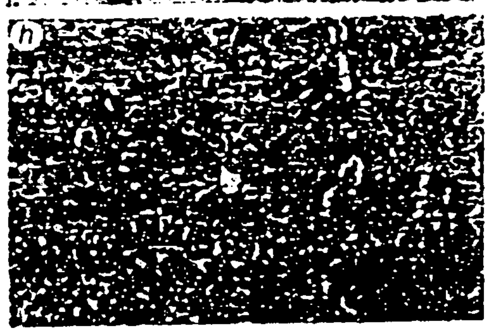

FIG 3h

FIG 3i

FIG 3j

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00595

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl^6:
C12N 015/11, 015/62, 015/85; A01K 067/27; A61K 048/00.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT and Chem Abs
See details in Electronic database box below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPM, JAPIO

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Derwent WPAT, USPM, JAPIO Keywords: Intercellular ( ) adhesion molecule# ( ) 2 and Chem Abs; Keyword: Intercellular ( ) adhesion ( ) molecule# ( ) 2 or ICAM ( )2 or ICAM ( )2/IT and (promoter# or regulat?/IT)
CHEM ABS: TGCTTATCAGCAGCT / TCATCAGTTTGCAGA / SQSN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Transplantation (1996), Cowan et al., vol 62: 155-160. "Targetting gene expresion to endothelial cells in transgenic mice using the human inter-cellular adhesion molecule 2 promoter".	1-21
X Y	The Journal of Immunology (1992), Xu et al., vol 149: 2650-2655. "Isolation, characterization, and expression of mouse ICAM-2 complementary and genomic DNA".	1-5 6-21
Y	AU 51172/90 (647017) (Centre for Blood Research, Inc.) published 13 September 1990 See entire document, in particular fig 2.	1-21

[X] Further documents are listed in the continuation of Box C   [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 1996	4 Nov 1996

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C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Doevendans et al., (1995), "Strategies for studying cardiovascular disease in transgenic and gene - targeted mice". In "Strategies in Transgenic animal Science". Monastersky, G.M. and Robl, J.M. eds, pg 107-144. See in particular pg 115-116.	1-21

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 96/00595

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	51172/90	CA	2012125	EP	391088	JP	3072430
		SU	1809837	US	5512660		

END OF ANNEX